

DNA ENCODING A PLANT DEOXYHYPUSINE SYNTHASE,  
A PLANT EUKARYOTIC INITIATION FACTOR 5A,  
TRANSGENIC PLANTS  
AND A METHOD FOR CONTROLLING SENESCENCE PROGRAMMED AND  
CELL DEATH IN PLANTS

This application is a continuation-in-part application of Serial No. 09/348,675, filed July 6, 1999 and of Serial No. 09/597,771.

**Field of the Invention**

The present invention relates to polynucleotides which encode plant polypeptides that exhibit senescence-induced expression. The invention also relates to transgenic plants containing the polynucleotides in antisense orientation and methods for controlling programmed cell death, including senescence, in plants. More particularly, the present invention relates to a senescence induced plant deoxyhypusine synthase gene and a senescence-induced eIF-5A gene whose expressions are induced by the onset of programmed cell death, including senescence, and the use of the deoxyhypusine synthase gene and eIF-5A gene, alone or in combination, to control programmed cell death and senescence in plants.

**Description of the Prior Art**

Senescence is the terminal phase of biological development in the life of a plant. It presages death and occurs at various levels of biological organization including the whole plant, organs, flowers and fruit, tissues and individual cells.

15           The onset of senescence can be induced by different factors both internal and external. Senescence is a complex, highly regulated developmental stage in the life of a plant or plant tissue, such as fruit, flowers and leaves. Senescence results in the coordinated breakdown of cell membranes and macromolecules and  
20           the subsequent mobilization of metabolites to other parts of the plant.

In addition to the programmed senescence which takes place during normal plant development, death of cells and tissues and ensuing remobilization of metabolites occurs as a coordinated response to external, environmental factors.

25           External factors that induce premature initiation of senescence, which is also

referred to as necrosis or apoptosis, include environmental stresses such as temperature, drought, poor light or nutrient supply, as well as pathogen attack. Plant tissues exposed to environmental stress also produce ethylene, commonly known as stress ethylene (Buchanan-Wollaston, V., 1997, J. Exp. Botany, 48:181-199; Wright,  
5 M., 1974, Plant, 120:63-69). Ethylene is known to cause senescence in some plants.

Senescence is not a passive process, but, rather, is an actively regulated process that involves coordinated expression of specific genes. During senescence,  
10 the levels of total RNA decrease and the expression of many genes is switched off (Bate et al., 1991, J. Exper. Botany, 42, 801-11; Hensel et al., 1993, The Plant Cell, 5, 553-64). However, there is increasing evidence that the senescence process depends on *de novo* transcription of nuclear genes. For example, senescence is blocked by inhibitors of mRNA and protein synthesis and enucleation. Molecular studies using mRNA from senescing leaves and green leaves for *in vitro* translation experiments show a changed pattern of leaf protein products in senescing leaves (Thomas et al, 1992, J. Plant Physiol., 139, 403-12). With the use of differential screening and subtractive hybridization techniques, many cDNA clones representing  
20 senescence-induced genes have been identified from a range of different plants, including both monocots and dicots, such as *Arabidopsis*, maize, cucumber, asparagus, tomato, rice and potato. Identification of genes that are expressed specifically during senescence is hard evidence of the requirement for *de novo* transcription for senescence to proceed.

25 The events that take place during senescence appear to be highly coordinated to allow maximum use of the cellular components before necrosis and death occur. Complex interactions involving the perception of specific signals and the induction of cascades of gene expression must occur to regulate this process. Expression of genes encoding senescence related proteins is probably regulated via  
30 common activator proteins that are, in turn, activated directly or indirectly by hormonal signals. Little is known about the mechanisms involved in the initial signaling or subsequent co-ordination of the process.

Coordinated gene expression requires factors involved in transcription and translation, including initiation factors. Translation initiation factor genes have been isolated and characterized in a variety of organisms, including plants. Eukaryotic translation initiation factor 5A (eIF-5A) is an essential protein factor approximately 17 KDa in size, which is involved in the initiation of eukaryotic cellular protein synthesis. It is characterized by the presence of hypusine [N-(4-amino-2-hydroxybutyl) lysine], a unique modified amino acid, known to be present only in eIF-5A. Hypusine is formed post-translationally via the transfer and hydroxylation of the butylamino group from the polyamine, spermidine, to the side chain amino group of a specific lysine residue in eIF-5A. Activation of eIF-5A involves transfer of the butylamine residue of spermidine to the lysine of eIF-5A, forming hypusine and activating eIF-5A. In eukaryotes, deoxyhypusine synthase (DHS) mediates the post-translational synthesis of hypusine in eIF-5A. A corresponding DHS gene has not been identified in plants, however, it is known that plant eIF-5A contains hypusine. The hypusine modification has been shown to be essential for eIF-5A activity *in vitro* using a methionyl-puromycin assay.

Hypusine is uniquely present in eIF-5A and is found in all eukaryotes, some archaebacteria (which appear to be related to eukaryota), but not in eubacteria. Moreover, the amino acid sequence of eIF-5A is highly conserved, especially in the region surrounding the hypusine residue, suggesting that eIF-5A and its activating protein, deoxyhypusine synthase, execute fundamentally important steps in eukaryotic cell physiology (Joe et al., JBC, 270:22386-22392, 1995). eIF-5A has been cloned from human, alfalfa, slime mold, *Neurospora crassa*, tobacco and yeast. It was originally identified as a general translation initiation factor based on its isolation from ribosomes of rabbit reticulocyte lysates and its *in vitro* activity in stimulating methionine-puromycin synthesis. However, more recent data indicate that eIF-5A is not a translation initiation factor for global protein synthesis, but rather serves to facilitate the translation of specific subsets of mRNA populations. For example, there is strong evidence from experiments with animal cells and yeast that one or more isoforms of eIF-5A play an essential role in mediating the translation of a subset of mRNAs involved in cell proliferation. There are two isoforms in yeast,

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and if both genes are silenced the cells are unable to divide (Park et al., Biol. Signals, 6:115-123, 1997). Similarly, silencing the expression of yeast deoxyhypusine synthase, which activates eIF-5A, blocks cell division. Indeed, inhibitors of deoxyhypusine synthase have been developed that are likely to have importance in the therapy of hyperproliferative conditions (Wolff, et al., JBC, 272:15865-15871, 1997). Other studies have indicated that another isoform of eIF-5A is essential for Rev function in HIV-1 replication or Rex function in HTLV V replication (Park, et al., Biol. Signals, 6:115-123, 1997). There are also at least two expressed eIF-5A genes in tobacco. Gene-specific probes indicate that although they are both expressed in all tissues examined, each gene has a distinctive expression pattern, presumably regulating the translation of specific transcripts (Chamot, et al., Nuc. Acids Res., 20:625-669, 1992).

Deoxyhypusine synthase has been purified from rat testis, HeLa cells, *Neurospora crassa* and yeast. The amino acid sequence of deoxyhypusine synthase is highly conserved, and the enzymes from different species share similar physical and catalytic properties and display cross-species reactivities with heterologous eIF-5A precursors (Park, et al., 6 Biol. Signals, 6:115-123, 1997).

Plant polyamines have been implicated in a wide variety of physiological effects including floral induction, embryogenesis, pathogen resistance, cell growth, differentiation and division (Evans et al., 1989, Annu. Rev. Plant Physiol. Plant Mol. Biol., 40, 235-269; and Galston, et al., 1990, Plant Physiol., 94, 406-10). It has been suggested that eIF-5A is the intermediary through which polyamines exert their effects (Chamot et al., 1992, Nuc. Acids Res., 20(4), 665-69).

Two genes encoding isoforms of eIF-5A from *Nicotiana* have been identified (NeIF-5A1 and NeIF-5A2) (Chamot et al., 1992, Nuc. Acids Res., 20(4), 665-69). The genes were shown to be very similar. However, they display differential patterns of expression. One gene appears to be constitutively expressed at the mRNA level, while the expression pattern of the other correlates with the presence or absence of photosynthetic activity. Based on gene structure and genomic

Southern mapping it has been suggested that there is a multigene family of NeIF-5A genes in tobacco. It is likely that there is an eIF-5A isoform that regulates translation of a subset of senescence/necrosis specific mRNA transcripts.

5 Presently, there is no widely applicable method for controlling the onset of programmed cell death (including senescence) caused by either internal or external, e.g., environmental stress, factors. It is, therefore, of interest to develop senescence modulating technologies that are applicable to all types of plants and that are effective at the earliest stages in the cascade of events leading to senescence.

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### **SUMMARY OF THE INVENTION**

This invention is based on the discovery and cloning of a full length cDNA clone encoding a tomato senescence-induced deoxyhypusine synthase (DHS), as well as full length senescence-induced DHS cDNA clones from *Arabidopsis* leaf and carnation petal. The nucleotide sequences and corresponding amino acid sequences are disclosed herein.

The invention is also based, in part, on the discovery and cloning of full length cDNA clones encoding a senescence-induced eIF-5A gene from tomato, *Arabidopsis* and carnation. The nucleotide sequence and corresponding amino acid sequence of each of the eIF-5A cDNA clones are disclosed herein.

25 The present invention provides a method for genetic modification of plants to control the onset of senescence, either age-related senescence or environmental stress-induced senescence. The senescence-induced DHS nucleotide sequences of the invention, fragments thereof, or combinations of such fragments, are introduced into a plant cell in reverse orientation to inhibit expression of the endogenous senescence-induced DHS gene, thereby reducing the level of  
30 endogenous senescence-induced DHS protein, and reducing and/or preventing activation of eIF-5A and ensuing expression of the genes that mediate senescence.

In another aspect of the invention, the senescence-induced eIF-5A nucleotide sequences of the invention, fragments thereof, or combinations of such fragments, are introduced into a plant cell in reverse orientation to inhibit expression of the endogenous senescence-induced eIF-5A gene, and thereby reduce the level of 5 endogenous senescence-induced eIF-5A protein, and reduce and/or prevent ensuing expression of the genes that mediate senescence. Alternatively, both DHS sequences and eIF-5A sequences can be used together to reduce the levels of endogenous DHS and eIF-5A proteins

10 In yet another aspect, the present invention is directed to a method for genetic modification of plants to control the onset of senescence, either age-related senescence or environmental stress-induced senescence *via* the introduction into a plant cell of a combination of senescence-induced eIF-5A nucleotide sequences of the invention and senescence-induced DHS nucleotide sequences of the invention in reverse orientation to inhibit expression of the endogenous senescence-induced eIF-5A gene and senescence-induced DHS gene, thereby reducing the level of endogenous senescence-induced DHS protein, and reducing and/or preventing activation of eIF-5A and ensuing expression of the genes that mediate senescence.

20 In yet another aspect, the present invention is directed to methods for genetic modification of plants to increase resistance to physiological disease (such as, but not limited to, blossom end rot) *via* the introduction into a plant cell of a combination of senescence-induced eIF-5A nucleotide sequences of the invention and/or senescence-induced DHS nucleotide sequences of the invention in reverse orientation to inhibit expression of the endogenous senescence-induced eIF-5A 25 gene and/or senescence-induced DHS gene, thereby reducing the level of endogenous senescence-induced DHS protein, and/or reducing and/or preventing activation of eIF-5A and ensuing expression of the genes that mediate senescence. In a particularly preferred aspect, the 3' end of the endogenous senescence-induced DHS in reverse orientation is introduced.

30 Using the methods of the invention, transgenic plants are generated and

monitored for growth, development and either natural or prematurely-induced senescence. Plants or detached parts of plants (e.g., cuttings, flowers, vegetables, fruits, seeds or leaves) exhibiting prolonged life or shelf life, (e.g., extended life of flowers, reduced fruit or vegetable spoilage, enhanced biomass, increased seed yield, increased resistance to physiological disease (e.g., blossom end rot), reduced seed aging and/or reduced yellowing of leaves) due to reduction in the level of 5 senescence-induced DHS, senescence-induced eIF-5A or both are selected as desired products having improved properties including reduced leaf yellowing, reduced petal abscission, reduced fruit and vegetable spoilage during shipping and 10 storage. These superior plants are propagated. Similarly, plants exhibiting increased resistance to environmental stress, e.g., decreased susceptibility to low temperature (chilling), drought, infection, etc., and/ or increased resistance to pathogens and/or physiological disease, are selected as superior products.

In one aspect, the present invention is directed to an isolated DNA molecule encoding senescence-induced DHS, wherein the DNA molecule hybridizes with SEQ ID NO:1, or a functional derivative of the isolated DNA molecule which hybridizes with SEQ ID NO:1. In one embodiment of this aspect of the invention, the isolated DNA molecule has the nucleotide sequence of SEQ ID NO:1, i.e., 100% complementarity (sequence identity) to SEQ ID NO:1.

The present invention also is directed to an isolated DNA molecule encoding 25 senescence-induced DHS, wherein the DNA molecule hybridizes with SEQ ID NO:9, or a functional derivative of the isolated DNA molecule which hybridizes with SEQ ID NO:9. In one embodiment of this aspect of the invention, the isolated DNA molecule has the nucleotide sequence of SEQ ID NO:9, i.e., 100% complementarity (sequence identity) to SEQ ID NO:9.

The present invention also is directed to an isolated DNA molecule encoding 30 senescence-induced eIF-5A, wherein the DNA molecule hybridizes with SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15 or a functional derivative of the isolated DNA molecule which hybridizes with SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15. In

one embodiment of this aspect of the invention, the isolated DNA molecule has the nucleotide sequence of SEQ ID NO:11, SEQ ID NO:13, or SEQ ID NO:15, i.e., 100% complementarity (sequence identity) to SEQ ID NO:11, SEQ ID NO:13 or SEQ ID NO:15.

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In another embodiment of the invention, there is provided an isolated protein encoded by a DNA molecule as described herein above, or a functional derivative thereof. A preferred protein has the amino acid sequence of SEQ ID NO:2, or is a functional derivative thereof. Another preferred protein has the amino acid sequence of SEQ ID NO:10, or is a functional derivative thereof. Other preferred proteins of the invention have the amino acid sequence of SEQ ID NO:12, SEQ ID NO:14 or SEQ ID NO: 16.

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Also provided herein is an antisense oligonucleotide or polynucleotide encoding an RNA molecule which is complementary to a corresponding portion of an RNA transcript of a DNA molecule described herein above, wherein the oligonucleotide or polynucleotide hybridizes with the RNA transcript such that expression of endogenous senescence-induced DHS is altered. In another embodiment of this aspect of the invention, the antisense oligonucleotide or polynucleotide is an RNA molecule that hybridizes to a corresponding portion of an RNA transcript of a DNA molecule described herein above, such that expression of endogenous senescence-induced eIF-5A is altered. The antisense oligonucleotide or polynucleotide can be full length or preferably has about six to about 100 nucleotides.

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The antisense oligonucleotide or polynucleotide may be substantially complementary to a corresponding portion of one strand of a DNA molecule encoding senescence-induced DHS, wherein the DNA molecule encoding senescence-induced DHS hybridizes with SEQ ID NO:1, SEQ ID NO: 5, SEQ ID NO: 9, or with a combination thereof, or is substantially complementary to at least a corresponding portion of an RNA sequence encoded by the DNA molecule encoding senescence-induced DHS. In one embodiment of the invention, the antisense

oligonucleotide or polynucleotide is substantially complementary to a corresponding portion of one strand of the nucleotide sequence SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:9 or with a combination thereof, or the RNA transcript transcribed from SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:9 or with a combination thereof. In another embodiment, the antisense oligonucleotide is substantially complementary to a corresponding portion of the 5' non-coding portion or 3' portion of one strand of a DNA molecule encoding senescence-induced DHS, wherein the DNA molecule hybridizes with SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:9 or with a combination thereof.

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Alternatively, the antisense oligonucleotide or polynucleotide may be substantially complementary to a corresponding portion of one strand of a DNA molecule encoding senescence-induced eIF-5A, wherein the DNA molecule encoding senescence-induced eIF-5A hybridizes with SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, or any combination thereof, or is substantially complementary to at least a corresponding portion of an RNA sequence transcribed from SEQ ID NO:11, SEQ ID NO:13 or SEQ ID NO:15. In one embodiment of the invention, the antisense oligonucleotide or polynucleotide is substantially complementary to a corresponding portion of one strand of the nucleotide sequence SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15 or a combination thereof, or the RNA transcript encoded is substantially complementary to a corresponding portion of an RNA sequence encoded by a DNA molecule encoding senescence-induced eIF-5A. In another embodiment, the antisense oligonucleotide is substantially complementary to a corresponding portion of the 5' non-coding region or 3' region of one strand of a DNA molecule encoding senescence-induced eIF-5A, wherein the DNA molecule hybridizes with SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15 or a combination thereof.

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The invention is further directed to a vector for transformation of plant cells, comprising

30 (a) an antisense oligo- or polynucleotide substantially complementary to (1) a corresponding portion of one strand of a DNA molecule encoding senescence-

induced DHS, wherein the DNA molecule encoding senescence-induced DHS hybridizes with SEQ ID NO:1, SEQ ID NO:5 or SEQ ID NO:9, or (2) a corresponding portion of an RNA sequence encoded by the DNA molecule encoding senescence-induced DHS; and

- 5           (b) regulatory sequences operatively linked to the antisense oligo- or polynucleotide such that the antisense oligo- or polynucleotide is expressed in a plant cell into which it is transformed.

10           The invention is further directed to a vector for transformation of plant cells, comprising

15           (a) an antisense oligo- or polynucleotide substantially complementary to (1) a corresponding portion of one strand of a DNA molecule encoding senescence-induced eIF-5A, wherein the DNA molecule encoding senescence-induced eIF-5A hybridizes with SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15 or (2) a corresponding portion of an RNA sequence encoded by the DNA molecule encoding senescence-induced eIF-5A; and

20           (b) regulatory sequences operatively linked to the antisense oligo- or polynucleotide such that the antisense oligo- or polynucleotide is expressed in a plant cell into which it is transformed.

25           The regulatory sequences include a promoter functional in the transformed plant cell, which promoter may be inducible or constitutive. Optionally, the regulatory sequences include a polyadenylation signal.

30           The invention also provides a plant cell transformed with a vector or combination of vectors as described above, a plantlet or mature plant generated from such a cell, or a plant part of such a plantlet or plant.

35           The present invention is further directed to a method of producing a plant having a reduced level of senescence-induced DHS, senescence-induced eIF-5A or both compared to an unmodified plant, comprising:

- (1) transforming a plant with a vector or combination of vectors as described

above;

(2) allowing the plant to grow to at least a plantlet stage;

5 (3) assaying the transformed plant or plantlet for altered senescence-induced DHS activity and/or eIF-5A activity and/or altered senescence and/or altered environmental stress-induced senescence and/or pathogen-induced senescence and/or ethylene-induced senescence; and

10 (4) selecting and growing a plant having altered senescence-induced DHS activity and/or reduced eIF-5A and/or altered senescence and/or altered environmental stress-induced senescence and/or altered pathogen-induced senescence and/or ethylene-induced senescence compared to a non-transformed plant.

Plants produced as above, or progeny, hybrids, clones or plant parts preferably exhibit reduced senescence-induced DHS expression, reduced senescence-induced eIF-5A activity, or both and delayed senescence and/or delayed stress-induced senescence and/or pathogen-induced senescence and/or ethylene-induced senescence.

This invention is further directed to a method of inhibiting expression of endogenous senescence-induced DHS in a plant cell, said method comprising:

(1) integrating into the genome of a plant a vector comprising

25 (A) an antisense oligo- or polynucleotide complementary to (I) at least a portion of one strand of a DNA molecule encoding endogenous senescence-induced DHS, wherein the DNA molecule encoding the endogenous senescence-induced DHS hybridizes with SEQ ID NO:1, SEQ ID NO:5 and/or SEQ ID NO.9, or (ii) at least a portion of an RNA sequence encoded by the endogenous senescence-induced DHS gene; and

30 (B) regulatory sequences operatively linked to the antisense oligo- or polynucleotide such that the antisense oligo- or polynucleotide is expressed; and

(2) growing said plant, whereby said antisense oligo- or polynucleotide is transcribed and the transcript binds to said endogenous RNA whereby expression of said senescence-induced DHS gene is inhibited.

This invention is further directed to a method of inhibiting expression of endogenous senescence-induced eIF-5A in a plant cell, said method comprising:

- (1) integrating into the genome of a plant a vector comprising
  - (A) an antisense oligo- or polynucleotide complementary to (I) a corresponding portion of one strand of a DNA molecule encoding endogenous senescence-induced eIF-5A, wherein the DNA molecule encoding the endogenous senescence-induced eIF-5A hybridizes with SEQ ID NO:11, SEQ ID NO:15, SEQ ID NO:17 or a combination thereof, or (ii) at least a portion of an RNA sequence encoded by the endogenous senescence-induced eIF-5A gene; and
  - (B) regulatory sequences operatively linked to the antisense oligo- or polynucleotide such that the antisense oligo- or polynucleotide is expressed; and
- (2) growing said plant, whereby said antisense oligo- or polynucleotide is transcribed and the transcript binds to said endogenous RNA whereby expression of said senescence-induced eIF-5A gene is inhibited.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the nucleotide sequence of the senescence-induced tomato leaf DHS cDNA sequence (SEQ ID NO:1) and the derived amino acid sequence (SEQ ID NO. 2) obtained from a tomato leaf cDNA library.

Figure 2A depicts the nucleotide sequence of an *Arabidopsis* DHS gene obtained by aligning the tomato DHS sequence with unidentified genomic sequences in the *Arabidopsis* gene bank (<http://genome-www.stanford.edu/Arabidopsis/>) (SEQ ID NO:5). The gaps between amino acid sequences are predicted introns. Figure 2B depicts the derived *Arabidopsis* DHS amino acid sequence (SEQ ID NO:6). Figure 2C depicts the nucleotide sequence of a 600 base pair *senescence-induced Arabidopsis* DHS cDNA obtained by PCR. Figure 2D depicts the derived amino acid sequence of the senescence-induced *Arabidopsis* DHS cDNA fragment.

Figure 3 is an alignment of the derived full length tomato leaf senescence-induced DHS amino acid sequence (SEQ ID NO. 2) and the derived full length

Arabidopsis senescence-induced DHS amino acid sequence with sequences of DHS proteins of human, yeast, fungi, and Archaeobacteria. Identical amino acids among three or four of the sequences are boxed.

5           Figure 4 is a restriction map of the tomato DHS cDNA.

Figure 5 is a Southern blot of genomic DNA isolated from tomato leaves and probed with  $^{32}\text{P}$ -dCTP-labeled full length tomato senescence-induced DHS cDNA.

10          Figure 6 is a Northern blot of RNA isolated from tomato flowers at different stages of development. Figure 6A is the ethidium bromide stained gel of total RNA. Each lane contains 10  $\mu\text{g}$  RNA. Figure 6B is an autoradiograph of the Northern blot probed with  $^{32}\text{P}$ -dCTP-labeled full length tomato senescence-induced DHS cDNA.

15          Figure 7 is a Northern blot of RNA isolated from tomato fruit at various stages of ripening that was probed with  $^{32}\text{P}$ -dCTP-labelled full length tomato senescence-induced DHS cDNA. Each lane contains 10  $\mu\text{g}$  RNA.

20          Figure 8 is a Northern blot of RNA isolated from tomato leaves that had been drought-stressed by treatment with 2 M sorbitol for six hours. Each lane contains 10  $\mu\text{g}$  RNA. The blot was probed with  $^{32}\text{P}$ -dCTP-labelled full length tomato senescence-induced DHS cDNA.

25          Figure 9 is a Northern blot of RNA isolated from tomato leaves that had been exposed to chilling temperature. Figure 9A is the ethidium bromide stained gel of total RNA. Each lane contained 10  $\mu\text{g}$  RNA. Figure 9B is an autoradiograph of the Northern blot probed with  $^{32}\text{P}$ -dCTP-labelled full length tomato senescence-induced DHS cDNA. Figure 9C shows corresponding leakage data measured as conductivity of leaf diffusates.

30          Figure 10 is the carnation DHS full-length (1384 base pairs) cDNA clone nucleotide sequence (SEQ ID NO: 9) not including the PolyA tail and 5' end non-

coding region. The derived amino acid sequence is shown below the nucleotide sequence (373 amino acids). (SEQ ID NO:10)

5       Figure 11 is a Northern blot of total RNA from senescing *Arabidopsis* leaves probed with  $^{32}\text{P}$ -dCTP-labelled full-length *Arabidopsis* senescence-induced DHS cDNA. The autoradiograph is at the top, the ethidium stained gel below.

10      Figure 12 is a Northern blot of total RNA isolated from petals of carnation flowers at various stages. The blot was probed with  $^{32}\text{P}$ -dCTP-labelled full-length carnation senescence-induced DHS cDNA. The autoradiograph is at the top, the ethidium stained gel below.

15      Figure 13 is the nucleotide (top) (SEQ ID NO:11) and derived amino acid (bottom) (SEQ ID NO:12) sequence of the tomato fruit senescence-induced eIF-5A gene.

20      Figure 14 is the nucleotide (top) (SEQ ID NO:13) and derived amino acid (bottom) (SEQ ID NO:14) sequence of the carnation senescence-induced eIF-5A gene.

25      Figure 15 is the nucleotide (top) (SEQ ID NO:15) and derived amino acid (bottom) (SEQ ID NO:16) sequence of the *Arabidopsis* senescence-induced eIF-5A gene.

30      Figure 16 is a Northern blot of total RNA isolated from leaves of *Arabidopsis* plants at various developmental stages. The blot was probed with  $^{32}\text{P}$ -dCTP-labelled full-length *Arabidopsis* senescence-induced DHS cDNA and full-length senescence-induced eIF-5A. The autoradiograph is at the top, the ethidium stained gel below.

35      Figure 17 is a Northern blot of total RNA isolated from tomato fruit at breaker (BK), red-firm (RF) and red-soft (RS) stages of development. The blot was probed

with  $^{32}\text{P}$ -dCTP-labelled full-length senescence-induced DHS cDNA and full-length senescence-induced eIF-5A. DHS and eIF-5A are up-regulated in parallel in red-soft fruit coincident with fruit ripening. The autoradiograph is at the top, the ethidium stained gel below.

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Figure 18 is a Northern blot of total RNA isolated from leaves of tomato that were treated with sorbitol to induce drought stress. C is control; S is sorbitol treated. The blot was probed with  $^{32}\text{P}$ -dCTP-labelled full-length senescence-induced DHS cDNA and full-length senescence-induced eIF-5A. Both eIF-5A and DHS are up-regulated in response to drought stress. The autoradiograph is at the top, the ethidium stained gel below.

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Figure 19 is a Northern blot of total RNA isolated from flower buds and open senescing flowers of tomato plants. The blot was probed with  $^{32}\text{P}$ -dCTP-labelled full-length senescence-induced DHS cDNA and full-length senescence-induced eIF-5A. Both eIF-5A and DHS are up-regulated in open/senescing flowers. The autoradiograph is at the top, the ethidium stained gel below.

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Figure 20 is a Northern blot of total RNA isolated from chill-injured tomato leaves. The blot was probed with  $^{32}\text{P}$ -dCTP-labelled full-length senescence-induced DHS cDNA and full-length senescence-induced eIF-5A. Both eIF-5A and DHS are up-regulated with the development of chilling injury during rewarming. The autoradiograph is at the top, the ethidium stained gel below.

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Figure 21 is a photograph of 3.1 week old *Arabidopsis* wild-type (left) and transgenic plants expressing the 3'-end of the senescence DHS gene (sequence shown in Figure 36) in antisense orientation showing increased leaf size in the transgenic plants.

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Figure 22 is a photograph of 4.6 week old *Arabidopsis* wild-type (left) and transgenic plants expressing the 3'-end of the senescence DHS gene (sequence shown in Figure 36) in antisense orientation showing increased leaf size in the

transgenic plants.

Figure 23 is a photograph of 5.6 week old *Arabidopsis* wild-type (left) and transgenic plants expressing the 3'-end of the senescence DHS gene (sequence shown in Figure 36) in antisense orientation showing increased leaf size in the transgenic plants.

Figure 24 is a photograph of 6.1 week old *Arabidopsis* wild-type (left) and transgenic plants expressing the 3'-end of the senescence DHS gene (sequence shown in Figure 36) in antisense orientation showing increased size of transgenic plants.

Figure 25 is a graph showing the increase in seed yield from three  $T_1$  transgenic *Arabidopsis* plant lines expressing the senescence-induced DHS gene in antisense orientation. Seed yield is expressed as volume of seed. SE for n=30 is shown for wild-type plants.

Figure 26 is a photograph of transgenic tomato plants expressing the 3'-end of the senescence DHS gene (sequence shown in Figure 36) in antisense orientation (left) and wild-type plants (right) showing increased leaf size and increased plant size in the transgenic plants. The photograph was taken 18 days after transfer of the plantlets to soil.

Figure 27 is a photograph of transgenic tomato plants expressing the 3'-end of the senescence DHS gene (sequence shown in Figure 36) in antisense orientation (left) and wild-type plants (right) showing increased leaf size and increased plant size in the transgenic plants. The photograph was taken 32 days after transfer of the plantlets to soil.

Figures 28 through 35 are photographs of tomato fruit from wild-type (top panels) and transgenic plants expressing the full-length senescence DHS gene in antisense orientation (bottom panels). Fruit were harvested at the breaker stage of

development and ripened in a growth chamber. Days after harvest are indicated in the upper left corner of each panel.

5       Figure 36 is the nucleotide (top) (SEQ ID NO:30) and derived amino acid (bottom) sequence of the 3'-end of the *Arabidopsis* senescence-induced DHS gene used in antisense orientation to transform plants.

10      Figure 37 is the nucleotide (top) (SEQ ID NO:31) and derived amino acid (bottom) sequence of the 3'-end of the tomato senescence-induced DHS gene used in antisense orientation to transform plants.

Figure 38 is the nucleotide (top) (SEQ ID NO:26) and derived amino acid (bottom) sequence of a 600 base pair *Arabidopsis* senescence-induced DHS probe used to isolate the full-length *Arabidopsis* gene.

Figure 39 is the nucleotide (top) (SEQ ID NO:27) and derived amino acid (bottom) sequence of the 483 base pair carnation senescence-induced DHS probe used to isolate the full-length carnation gene.

20      Figure 40 (a) and (b) are photographs of tomato fruits from transgenic tomato plants expressing the 3'-end of the senescence DHS gene (sequence shown in Figure 37) in antisense orientation (right) and tomato fruits from wild-type plants (left). While the wild-type fruit exhibits blossom end rot, the transgenic fruit does not.

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#### **DETAILED DESCRIPTION OF THE INVENTION**

Methods and compositions are provided for altering the expression of 30 senescence-induced DHS gene(s), senescence-induced eIF-5A gene(s) or both in plant cells. Alteration of expression of senescence-induced DHS and senescence-induced eIF-5A, either alone or in combination, in plants results in delayed onset of senescence and improved resistance to environmental stress and pathogens, thus extending the plant shelf-life and/or growth period.

A full length cDNA sequence encoding a tomato DHS gene exhibiting senescence-induced expression has been isolated by reverse transcriptase mediated polymerase chain reaction (RT-PCR) using RNA isolated from chill-injured tomato leaves as a template and using the RT-PCR product to screen a chill-injured, 5 sorbitol-treated tomato leaf cDNA library. Polynucleotide probes corresponding to selected regions of the isolated tomato leaf cDNA sequence as well as the full length tomato leaf cDNA were used to determine the presence of mRNA encoding the DHS gene in environmentally stressed (chilled) tomato leaves, (dehydrated) sorbitol-treated tomato leaves, ripening tomato fruit and senescent tomato blossoms.

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Primers designed from an *Arabidopsis* DHS genomic clone were used to generate a polymerase chain reaction (PCR) product using a senescent *Arabidopsis* leaf cDNA library as template. The *Arabidopsis* nucleotide sequence has 73% nucleotide sequence identity and 81% amino acid sequence identity with the corresponding sequence of the senescence-induced tomato DHS.

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The senescence-induced tomato DHS gene of the present invention was isolated by using RT-PCR. The upstream primer used to isolate the tomato DHS gene is a 24 nucleotide primer: 5' AG TCT AGA AGG TGC TCG TCC TGA T 3' (SEQ ID NO. 3); the downstream primer contains 34 nucleotides: 5' G ACT GCA GTC GAC ATC GAT (T)<sub>15</sub> 3' (SEQ ID NO. 4). Using 100 pmol of the downstream primer, a first strand of cDNA was isolated using standard RT-PCR. The first strand was then used as template in a RT-PCR, using both the upstream and downstream primers. Separation of the RT-PCR products on an agarose gel revealed the presence of three distinct bands ranging in size from 1.5 kb to 600 bp. The three fragments were subcloned into the plasmid vector, pBluescript<sup>TM</sup> (Stratagene Cloning Systems, LaJolla, CA) using XbaI and SalI cloning sites present in the upstream and downstream primers, respectively, and sequenced. The sequences of the fragments were compared and aligned with sequences present in the GeneBank data base. The results showed the 1.5 kb and 1 kb fragments to be tomato DHS sequence. The 600 bp fragment also aligned with human, yeast and *Neurospora* DHS sequences.

The 600 bp RT-PCR fragment was used to screen a tomato (cv. Match F1 hybrid) cDNA library made from RNA obtained from tomato leaves that had been treated with 2 M sorbitol for six hours to induce dehydration. The cDNA library was constructed using a  $\lambda$ Zap<sup>TM</sup> (Stratagene Cloning Systems, LaJolla, CA) cDNA library kit. Three identical positive full-length cDNA clones corresponding to the senescence-induced DHS gene were obtained and sequenced. The nucleotide sequence of the senescence-induced DHS cDNA clone is shown in SEQ ID NO:1. The cDNA clone encodes a 381 amino acid polypeptide (SEQ ID NO: 2) having a calculated molecular mass of 42.1KDa.

Based on the expression pattern of the gene in developing and stressed tomato flowers, fruit and leaves, it is involved in senescence.

The tomato DHS cDNA sequence was aligned with unidentified genomic sequences in the *Arabidopsis thaliana* genome bank (<http://genome-www.stanford.edu/Arabidopsis>). The results showed alignment with an unidentified *Arabidopsis* genomic sequence (AB107060). The alignment information was used to identify an open reading frame in the *Arabidopsis* sequence and generate predicted amino acid sequence therefrom. The resulting nucleotide and amino acid sequences of the aligned *Arabidopsis* DHS gene are designated as SEQ ID NO. 5 (Figure 2A) and SEQ ID NO. 6, respectively.

Two primers based on short regions of the identified *Arabidopsis* DHS sequence were generated: primer 1, 5' GGTGGTGTTGAGGAAGATC 3' (SEQ ID NO. 7); and primer 2, 5' GGGTGCACGCCCTGATGAAGC 3' (SEQ ID NO. 8). An *Arabidopsis* senescent leaf cDNA library was used as template for the two primers in a standard PCR. A 600 bp PCR product was isolated and sequenced and shown to have an identical sequence as that of the corresponding fragment of the genomic DHS sequence.

The full-length senescence-induced tomato DHS cDNA clone was also used to isolate full-length senescence-induced *Arabidopsis* and carnation DHS cDNA

clones. The *Arabidopsis* and carnation DHS cDNA clones were isolated by screening a senescent *Arabidopsis* leaf cDNA library and a senescing carnation petal cDNA library, respectively, using the full-length tomato DHS cDNA clone as probe. cDNA clones obtained from the cDNA libraries were then sequenced. The 5 nucleotide sequence of the *Arabidopsis* full-length cDNA clone isolated in this manner has the same sequence as the coding region of the *Arabidopsis* genomic sequence identified as encoding *Arabidopsis* DHS by alignment with the tomato cDNA sequence. (Figure 2A, SEQ ID NO: 5). The nucleotide sequence of the full-length carnation petal senescence-induced DHS clone and derived amino acid 10 sequence are shown in Figure 10 (SEQ ID NO:9 and SEQ ID NO:10, respectively).

Thus, the cDNA sequences of the invention, encoding DHS from tomato, carnation and *Arabidopsis* can be used as probe in a similar manner to isolate DHS genes from other plants, which can then be used to alter senescence in transgenic plants.

The senescence-induced DHS gene appears to be a member of a DHS gene family. Genomic Southern blot analysis of tomato leaf DNA was carried out using genomic DNA extracted from a hybrid plant. The DNA was cut with various restriction enzymes that recognize a single site within the coding region of the DHS gene or which do not recognize any sites within the open reading frame of the DHS gene. A restriction map for tomato DHS is shown in Figure 4.

25 Restriction enzyme digested tomato leaf genomic DNA was probed with <sup>32</sup>P-dCTP-labeled full length tomato DHS cDNA. Hybridization under high stringency conditions revealed hybridization of the full length cDNA probe to two to three restriction fragments for each restriction enzyme digested DNA sample. Of particular note, when tomato leaf genomic DNA was digested with XbaI and EcoRI, which have restriction sites within the open reading frame of DHS (Figure 4), more than two restriction fragments were detectable in the Southern blot (Figure 5). Genomic DNA from cv Match F1, a hybrid variety, and the homozygous line, UCT5, yielded the same pattern of restriction fragments. These results suggest that there 30

are two or more isoforms of the DHS gene in tomato plants. As shown in Figure 3, the DHS gene is highly conserved across species and so it would be expected that there is a significant amount of conservation between isoforms within any species.

5 Northern blots of tomato flower total RNA probed with the full length tomato cDNA show that the expression of the senescence-induced DHS gene is significantly induced in tomato blossoms, but expression is barely detectable in the buds (Figure 6). Northern blot analysis of DHS expression during various developmental stages of tomato fruit demonstrate that the DHS gene is expressed 10 at low levels in breaker and pink fruit, whereas DHS expression in red (ripe) tomato fruit is significantly enhanced (Figure 7).

Northern blot analyses also demonstrate that the senescence-induced DHS gene is induced by environmental stress conditions, e.g., dehydration (Figure 8) and chilling (Figure 9). Tomato leaves that had been treated with 2 M sorbitol to induce dehydration demonstrate induction of DHS expression in the dehydrated leaves compared to non-treated leaves (Figure 8). Plants that have been exposed to chilling temperatures and returned to ambient temperature show induced expression of the senescence-induced DHS gene coincident with the development of chilling injury symptoms (e.g., leakiness) (Figure 9). The overall pattern of gene expression in tomato plants and various plant tissues, e.g., leaves, fruit and flowers, demonstrates that the DHS gene of the invention is involved in the initiation of senescence in these plants and plant tissues.

25 Similar results in terms of induction of DHS gene expression are observed with the onset of leaf senescence in *Arabidopsis* and petal senescence in carnation. Northern blot analyses of *Arabidopsis* leaf total RNA isolated from plants of various ages show that the expression of the senescence-induced DHS gene is not evident in young (five-week-old plants), but begins to appear at about six weeks. Expression 30 of the DHS gene is significantly induced by seven weeks. Northern blot analysis indicates that the *Arabidopsis* DHS gene is significantly enhanced as the plant ages. (Figure 11).

Northern blot analyses also demonstrate that the DHS gene is similarly regulated in flowering plants, such as the carnation. (Figure 12) Northern blot analyses of total RNA isolated from petals of carnation flowers of various ages show that the expression of carnation DHS is significantly induced in petals from flowers  
5 that have symptoms of age-induced senescence such as petal inrolling, which is the first morphological manifestation of senescence, but expression is much lower in tight-bud flowers. Petals from carnation flowers that are just beginning to open have significantly more DHS expression than flowers in the tight-bud stage, and petals from flowers that are fully open also show enhanced expression of DHS.

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Thus, it is expected that by substantially repressing or altering the expression of the senescence-induced DHS gene in plant tissues, deterioration and spoilage can be delayed, increasing the shelf-life of perishable fruits, flowers, and vegetables, and plants and their tissues can be rendered more stress-tolerant and pathogen resistant. This can be achieved by producing transgenic plants in which the DHS cDNA or an oligonucleotide fragment thereof is expressed in the antisense configuration in fruits, flowers, leaves and vegetables, preferably using a constitutive promoter such as the CaMV 35S promoter, or using a tissue-specific or senescence/stress-inducible promoter.

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Another gene, eIF-5A, which is involved in the induction of senescence related morphological changes in plants has also been isolated and sequenced herein and like the DHS, it can be used to alter senescence and senescence-related processes in plants, preferably, by introduction in antisense orientation into plants.

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A full-length senescence-induced eIF-5A cDNA clone was isolated from each of ripening tomato fruit, senescent *Arabidopsis* leaf and senescent carnation flower cDNA libraries. The nucleotide and derived amino acid sequences of each of the full length clones is shown in Figures 13 (tomato senescence-induced eIF-5A), 14 (carnation senescence-induced eIF-5A) and 15 (*Arabidopsis* senescence-induced eIF-5A). The nucleotide sequence of each of these cDNA clones is also shown as SEQ ID NO: 11 (tomato) (Figure 13), SEQ ID NO:13 (carnation) (Figure 14) and SEQ ID NO:15 (*Arabidopsis*) (Figure 15). The derived amino acid sequence of each

of the genes is shown as SEQ ID NO:12 (Figure 13), SEQ ID NO:14 (Figure 14) and SEQ ID NO:16 (Figure 15), respectively.

As is the case with the DHS gene sequences described herein, the eIF-5A sequence of the present invention can be used to isolate eIF-5A genes from other plants. The isolated eIF-5A sequences can be used to alter senescence and senescence-related processes in plants. Isolation of eIF-5A sequences from plants can be achieved using art known methods, based on sequences similarities of at least about 70% across species.

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Parallel induction of eIF-5A and DHS occurs in plants during senescence. Northern blot analyses demonstrate that eIF-5A is upregulated in parallel with DHS at the onset of both natural and stress-induced senescence. (Figures 16 through 20) For example, Northern blot analyses of total RNA isolated from leaves of *Arabidopsis* plants at various ages demonstrate that from the time leaf senescence is evident in the plant the expression of eIF-5A is induced and expression is significantly enhanced as senescence progresses. In fruit bearing plants, such as tomato, eIF-5A and DHS are upregulated in parallel in red-soft fruit coincident with the onset of fruit softening and spoilage. (Figure 17)

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Northern blot analysis also demonstrates that eIF-5A and DHS are upregulated in parallel in plants in response to environmental stress, such as drought (Figure 18) and chilling injury (Figure 20). Similarly, in flowering plants, eIF-5A and DHS are upregulated in parallel in open flowers and expression of both genes continues to be enhanced through the later stages of flowering.

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The cloned senescence-induced DHS gene, fragment(s) thereof, or cloned senescence-induced eIF-5A gene or fragment(s) thereof, or combinations of eIF-5A and DHS sequences, when introduced in reverse orientation (antisense) under control of a constitutive promoter, such as the fig wart mosaic virus 35S promoter, cauliflower mosaic virus promoter CaMV35S, double 35S promoter or MAS promoter, can be used to genetically modify plants and alter senescence in the

modified plants. Selected antisense sequences from other plants which share sufficient sequence identity with the tomato, *Arabidopsis* or carnation senescence-induced DHS genes or senescence-induced eIF-5A genes can be used to achieve similar genetic modification. One result of the genetic modification is a reduction in  
5 the amount of endogenous translatable senescence-induced DHS-encoding mRNA, eIF-5A-encoding mRNA or both. Consequently, the amount of senescence-induced DHS and/or senescence-induced eIF-5A produced in the plant cells is reduced, thereby reducing the amount of activated eIF-5A, which in turn reduces translation of senescence induced proteins, including senescence-induced lipase, senescence-  
10 induced proteases and senescence-induced nucleases. Senescence is thus inhibited or delayed, since *de novo* protein synthesis is required for the onset of senescence.

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For example, *Arabidopsis* plants transformed with vectors that express either the full-length or 3'- region of the *Arabidopsis* senescence-induced DHS gene (SEQ ID NO:26) (Figure 38) in antisense orientation, under regulation of a double 35S promoter exhibit increased biomass, e.g., larger leaf size and overall larger plant growth throughout all stages of growth, and delayed leaf senescence in comparison to control plants as shown in Figures 21 through 24.

25 The effect of reduced expression of the senescence-induced DHS gene brought about by expressing either the full-length or 3' coding region of the *Arabidopsis* senescence-induced DHS gene in antisense orientation in transgenic *Arabidopsis* plants is also seen as an increase in seed yield in the transformed plants. *Arabidopsis* plant lines expressing the antisense 3' non-coding region of the *Arabidopsis* senescence-induced DHS gene produce up to six times more seed than wild type plants. (Figure 25)

30 Similar results are obtained with tomato plants transformed with the 3' end of the tomato senescence-induced DHS gene (SEQ ID NO:27) in antisense orientation and under regulation of a double 35S promoter. Plants transformed with the 3' end of the gene in antisense orientation show increased leaf size and increased plant

size in comparison to control (non-transformed) tomato plants. (Figures 26 and 27)

Tomato plants transformed with the full length tomato senescence-induced DHS in antisense orientation produce fruit that exhibits delayed softening and spoilage in comparison to wild type plants. (Figures 28 through 35). Thus, the methods and sequences of the present invention can be used to delay fruit softening and spoilage, as well as to increase plant biomass and seed yield and in general, delay senescence in plants.

Tomato fruits of tomato plants transformed with vectors that express the 3'-region of the tomato senescence-induced DHS gene (SEQ ID NO:31) (Figure 37) in antisense orientation, under regulation of a double 35S promoter exhibit increased resistance to blossom end rot, a physiological disease, in comparison to control plants as shown in Figure 40.

The isolated nucleotide sequences of this invention can be used to isolate substantially complementary DHS and/or eIF-5A nucleotide sequence from other plants or organisms. These sequences can, in turn, be used to transform plants and thereby alter senescence of the transformed plants in the same manner as shown with the use of the isolated nucleotide sequences shown herein.

The genetic modifications obtained with transformation of plants with DHS, eIF-5A , fragments thereof or combinations thereof can effect a permanent change in levels of senescence-induced DHS, eIF-5A or both in the plant and be propagated in offspring plants by selfing or other reproductive schemes. The genetically altered plant is used to produce a new variety or line of plants wherein the alteration is stably transmitted from generation to generation. The present invention provides for the first time the appropriate DNA sequences which may be used to achieve a stable genetic modification of senescence in a wide range of different plants.

For the identification and isolation of the senescence-induced DHS gene and eIF-5A gene, in general, preparation of plasmid DNA, restriction enzyme

digestion, agarose gel electrophoresis of DNA, polyacrylamide gel electrophoresis of protein, PCR, RT-PCR, Southern blots, Northern blots, DNA ligation and bacterial transformation were carried out using conventional methods well-known in the art. See, for example, Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989. Techniques of nucleic acid hybridization are disclosed by Sambrook (Supra).

As used herein, the term "plant" refers to either a whole plant, a plant part, a plant cell or a group of plant cells. The type of plant which can be used in the methods of the invention is not limited and includes, for example, ethylene-sensitive and ethylene-insensitive plants; fruit bearing plants such as apricots, apples, oranges, bananas, grapefruit, pears, tomatoes, strawberries, avocados, etc.; vegetables such as carrots, peas, lettuce, cabbage, turnips, potatoes, broccoli, asparagus, etc.; flowers such as carnations, roses, mums, etc.; agronomic crop plants and forest species such as corn, rice, soybean, alfalfa and the like; and in general, any plant that can take up and express the DNA molecules of the present invention. It may include plants of a variety of ploidy levels, including haploid, diploid, tetraploid and polyploid. The plant may be either a monocotyledon or dicotyledon.

A transgenic plant is defined herein as a plant which is genetically modified in some way, including but not limited to a plant which has incorporated heterologous or homologous senescence-induced DHS DNA or modified DNA or some portion of heterologous senescence-induced DHS DNA or homologous senescence-induced DHS DNA into its genome. Alternatively a transgenic plant of the invention may have incorporated heterologous or homologous senescence-induced eIF-5A DNA or modified DNA or some portion of heterologous senescence-induced eIF-5A DNA or homologous senescence-induced eIF-5A DNA into its genome. Transgenic plants of the invention may have incorporated heterologous or homologous senescence-induced DHS and eIF-5A DNA or modified DNA or some portion of heterologous senescence-induced DHS and eIF-5A DNA or homologous senescence-induced DHS DNA or a combination of heterologous and homologous DHS and eIF-5A

sequences into its genome. The altered genetic material may encode a protein, comprise a regulatory or control sequence, or may be or include an antisense sequence or encode an antisense RNA which is antisense to the endogenous 5 senescence-induced DHS or eIF-5A DNA or mRNA sequence or portion thereof of the plant. A "transgene" or "transgenic sequence" is defined as a foreign gene or partial sequence which has been incorporated into a transgenic plant.

The term "hybridization" as used herein is generally used to mean hybridization of nucleic acids at appropriate conditions of stringency as would be 10 readily evident to those skilled in the art depending upon the nature of the probe sequence and target sequences. Conditions of hybridization and washing are well known in the art, and the adjustment of conditions depending upon the desired stringency by varying incubation time, temperature and/or ionic strength of the solution are readily accomplished. See, for example, Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Press, Cold Spring 15 Harbor, New York, 1989. The choice of conditions is dictated by the length of the sequences being hybridized, in particular, the length of the probe sequence, the relative G-C content of the nucleic acids and the amount of mismatches to be permitted. Low stringency conditions are preferred when partial hybridization 20 between strands that have lesser degrees of complementarity is desired. When perfect or near perfect complementarity is desired, high stringency conditions are preferred. For typical high stringency conditions, the hybridization solution contains 25 6X S.S.C., 0.01 M EDTA, 1X Denhardt's solution and 0.5% SDS. Hybridization is carried out at about 68°C for about 3 to 4 hours for fragments of cloned DNA and for about 12 to about 16 hours for total eukaryotic DNA. For lower stringencies the temperature of hybridization is reduced to about 42°C below the melting 30 temperature ( $T_m$ ) of the duplex. The  $T_m$  is known to be a function of the G-C content and duplex length as well as the ionic strength of the solution.

As used herein, the term "substantial sequence identity" or "substantial homology" is used to indicate that a nucleotide sequence or an amino acid sequence exhibits substantial structural or functional equivalence with another

nucleotide or amino acid sequence. Any structural or functional differences between sequences having substantial sequence identity or substantial homology will be *de minimis*; that is, they will not affect the ability of the sequence to function as indicated in the desired application. Differences may be due to inherent variations in codon usage among different species, for example. Structural differences are considered *de minimis* if there is a significant amount of sequence overlap or similarity between two or more different sequences or if the different sequences exhibit similar physical characteristics even if the sequences differ in length or structure. Such characteristics include, for example, ability to hybridize under defined conditions, or in the case of proteins, immunological crossreactivity, similar enzymatic activity, etc. Each of these characteristics can readily be determined by the skilled practitioner by art known methods.

DETAILED DESCRIPTION

Additionally, two nucleotide sequences are "substantially complementary" if the sequences have at least about 70 percent, more preferably, 80 percent and most preferably about 90 percent sequence similarity between them. Two amino acid sequences are substantially homologous if they have at least 50%, preferably 70% similarity between the active portions of the polypeptides.

As used herein, the phrase "hybridizes to a corresponding portion" of a DNA or RNA molecule means that the molecule that hybridizes, e.g., oligonucleotide, polynucleotide, or any nucleotide sequence (in sense or antisense orientation) recognizes and hybridizes to a sequence in another nucleic acid molecule that is of approximately the same size and has enough sequence similarity thereto to effect hybridization under appropriate conditions. For example, a 100 nucleotide long antisense molecule from the 3' coding or non-coding region of tomato DHS will recognize and hybridize to an approximately 100 nucleotide portion of a nucleotide sequence within the 3' coding or non-coding region, respectively of carnation DHS gene or any other plant DHS gene so long as there is about 70% or more sequence similarity between the two sequences. It is to be understood that the size of the "corresponding portion" will allow for some mismatches in hybridization such that the "corresponding portion" may be smaller or larger than the molecule which hybridizes

to it, for example 20-30% larger or smaller, preferably no more than about 12-15 % larger or smaller.

The term "functional derivative" of a nucleic acid (or poly- or oligonucleotide) is used herein to mean a fragment, variant, homolog, or analog of the gene or nucleotide sequence encoding senescence-induced DHS or senescence-induced eIF-5A. A functional derivative may retain at least a portion of the function of the senescence-induced DHS or eIF-5A encoding DNA which permits its utility in accordance with the invention. Such function may include the ability to hybridize under low stringency conditions with native tomato, *Arabidopsis* or carnation senescence-induced DHS or eIF-5A or substantially homologous DNA from another plant which encodes senescence-induced DHS or eIF-5A or with an mRNA transcript thereof, or, in antisense orientation, to inhibit the transcription and/or translation of plant senescence-induced DHS or eIF-5A mRNA, or the like.

A "fragment" of the gene or DNA sequence refers to any subset of the molecule, e.g., a shorter polynucleotide or oligonucleotide. A "variant" refers to a molecule substantially similar to either the entire gene or a fragment thereof, such as a nucleotide substitution variant having one or more substituted nucleotides, but which maintains the ability to hybridize with the particular gene or to encode mRNA transcript which hybridizes with the native DNA. A "homolog" refers to a fragment or variant sequence from a different plant genus or species. An "analog" refers to a non-natural molecule substantially similar to or functioning in relation to either the entire molecule, a variant or a fragment thereof.

By "altered expression" or "modified expression" of a gene, e.g., the senescence-induced DHS gene or senescence-induced eIF-5A gene, is meant any process or result whereby the normal expression of the gene, for example, that expression occurring in an unmodified fruit bearing, flowering or other plant, is changed in some way. As intended herein, alteration in gene expression is complete or partial reduction in the expression of the senescence-induced DHS gene or senescence-induced eIF-5A gene or both, but may also include a change in

the timing of expression, or another state wherein the expression of the senescence-induced DHS gene or senescence-induced eIF-5A gene or both differs from that which would be most likely to occur naturally in an unmodified plant or cultivar. A preferred alteration is one which results in reduction of senescence-induced DHS production, senescence-induced eIF-5A production or both by the plant compared to production in an unmodified plant.

In producing a genetically altered plant in accordance with this invention, it is preferred to select individual plantlets or plants by the desired trait, generally reduced senescence-induced DHS expression or production or reduced senescence-induced eIF-5A expression or both. Expression of senescence-induced DHS and senescence-induced eIF-5A can be determined, for example by observations of delayed or reduced senescence in transgenic plants. It is also possible to quantitate the activity of DHS and/or eIF-5A in transgenic plants in comparison to control (normal, non-transgenic) plants using known assays.

In order for a newly inserted gene or DNA sequence to be expressed, resulting in production of the protein which it encodes, or in the case of antisense DNA, to be transcribed, resulting in an antisense RNA molecule, the proper regulatory elements should be present in proper location and orientation with respect to the gene or DNA sequence. The regulatory regions may include a promoter, a 5'-non-translated leader sequence and a 3'-polyadenylation sequence as well as enhancers and other regulatory sequences.

Promoter regulatory elements that are useful in combination with the senescence-induced DHS gene to generate sense or antisense transcripts of the gene include any plant promoter in general, and more particularly, a constitutive promoter such as the fig wart mosaic virus 35S promoter, the cauliflower mosaic virus promoter, CaMV35S promoter, or the MAS promoter, or a tissue-specific or 30 senescence-induced promoter, such as the carnation petal GST1 promoter or the *Arabidopsis* SAG12 promoter (See, for example, J.C. Palaqui et al., *Plant Physiol.*, 112:1447-1456 (1996); Morton et al., *Molecular Breeding*, 1:123-132 (1995); Fobert

et al., Plant Journal, 6:567-577 (1994); and Gan et al., Plant Physiol., 113:313 (1997), incorporated herein by reference). Preferably, the promoter used in the present invention is a constitutive promoter, most preferably a double 35S promoter is used.

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Expression levels from a promoter which is useful for the present invention can be tested using conventional expression systems, for example by measuring levels of a reporter gene product, e.g., protein or mRNA in extracts of the leaves, flowers, fruit or other tissues of a transgenic plant into which the promoter/reporter gene have been introduced.

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The present invention provides antisense oligonucleotides and polynucleotides complementary to the gene encoding tomato senescence-induced DHS, carnation senescence-induced DHS, *Arabidopsis* senescence-induced DHS or complementary to a gene or gene fragment from another plant, which hybridizes with the tomato, carnation or *Arabidopsis* senescence-induced DHS gene under low to high stringency conditions. The present invention also provides antisense oligonucleotides and polynucleotides complementary to the gene encoding tomato senescence-induced eIF-5A, carnation senescence-induced eIF-5A, *Arabidopsis* senescence-induced eIF-5A or complementary to a gene or gene fragment from another plant, which hybridizes with the tomato, carnation or *Arabidopsis* senescence-induced eIF-5A gene under low to high stringency conditions. Such antisense oligonucleotides should be at least about six nucleotides in length to provide minimal specificity of hybridization and may be complementary to one strand of DNA or mRNA encoding the senescence-induced gene or a portion thereof, or to flanking sequences in genomic DNA which are involved in regulating senescence-induced DHS or eIF-5A gene expression. The antisense oligonucleotide may be as large as 100 nucleotides or more and may extend in length up to and beyond the full coding sequence for which it is antisense. The antisense oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single stranded or double stranded.

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The action of the antisense oligonucleotide may result in alteration, primarily inhibition, of senescence-induced DHS expression, senescence-induced eIF-5A expression or both in cells. For a general discussion of antisense see: Alberts, et al., Molecular Biology of the Cell, 2nd ed., Garland Publishing, Inc. New York, New York ,1989 (in particular pages 195-196, incorporated herein by reference).

The antisense oligonucleotide may be complementary to any corresponding portion of the senescence-induced DHS or eIF-5A gene. In one embodiment, the antisense oligonucleotide may be between 6 and 100 nucleotides in length, and may be complementary to the 5'-non-coding or sequences within the 3'- end of the senescence-induced DHS or eIF-5A sequence, for example. Antisense oligonucleotides primarily complementary to 5'-non-coding sequences are known to be effective inhibitors of expression of genes encoding transcription factors. Branch, M.A., Molec. Cell Biol., 13:4284-4290 (1993).

Preferred antisense oligonucleotides are substantially complementary to a portion of the mRNA encoding senescence-induced DHS or senescence-induced eIF-5A, the portion of the mRNA being approximately the same size as the antisense oligonucleotide. For example, introduction of the full length cDNA clone encoding senescence-induced DHS or eIF-5A in an antisense orientation into a plant is expected to result in successfully altered senescence-induced DHS and/or eIF-5A gene expression. Moreover, as demonstrated in Figures 21-35 introduction of partial sequences, targeted to specific portions of the senescence-induced DHS gene or senescence-induced eIF-5A gene or both, can be equally effective.

The minimal amount of homology required by the present invention is that sufficient to result in sufficient complementarity to provide recognition of the specific target RNA or DNA and inhibition or reduction of its translation or function while not affecting function of other RNA or DNA molecules and the expression of other genes. While the antisense oligonucleotides of the invention comprise sequences complementary to a corresponding portion of an RNA transcript of the senescence-induced DHS gene or senescence-induced eIF-5A gene, absolute complementarity,

although preferred is not required. The ability to hybridize may depend on the length of the antisense oligonucleotide and the degree of complementarity. Generally, the longer the hybridizing nucleic acid, the more base mismatches with the senescence-induced DHS target sequence it may contain and still form a stable duplex. One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting temperature of the hybridized complex, for example.

The antisense RNA oligonucleotides may be generated intracellularly by transcription from exogenously introduced nucleic acid sequences. The antisense molecule may be delivered to a cell by transformation or transfection or infection with a vector, such as a plasmid or virus into which is incorporated DNA encoding the antisense senescence-induced DHS sequence operably linked to appropriate regulatory elements, including a promoter. Within the cell the exogenous DNA sequence is expressed, producing an antisense RNA of the senescence-induced DHS gene.

Vectors can be plasmids, preferably, or may be viral or other vectors known in the art to replicate and express genes encoded thereon in plant cells or bacterial cells. The vector becomes chromosomally integrated such that it can be transcribed to produce the desired antisense senescence-induced DHS RNA. Such plasmid or viral vectors can be constructed by recombinant DNA technology methods that are standard in the art. For example, the vector may be a plasmid vector containing a replication system functional in a prokaryotic host and an antisense oligonucleotide or polynucleotide according to the invention. Alternatively, the vector may be a plasmid containing a replication system functional in *Agrobacterium* and an antisense oligonucleotide or polynucleotide according to the invention. Plasmids that are capable of replicating in *Agrobacterium* are well known in the art. See, Miki, et al., Procedures for Introducing Foreign DNA Into Plants, Methods in Plant Molecular Biology and Biotechnology,, Eds. B.R. Glick and J.E. Thompson. CRC Press (1993), PP. 67-83.

The tomato DHS gene was cloned in antisense orientation into a plasmid vector in the following manner. The pCD plasmid, which is constructed from a pUC18 backbone and contains the 35S promoter from cauliflower mosaic virus (CaMV) followed by a multiple cloning site and an octapine synthase termination sequence was used for cloning the tomato DHS gene. The pCd-DHS (antisense) plasmid was constructed by subcloning the full length tomato DHS gene in the antisense orientation into the pCD plasmid using Xhol and SacI restriction sites.

An oligonucleotide, preferably between about 6 and about 100 nucleotides in length and complementary to the target sequence of senescence-induced DHS or senescence-induced eIF-5A gene, may be prepared by recombinant nucleotide technologies or may be synthesized from mononucleotides or shorter oligonucleotides, for example. Automated synthesizers are applicable to chemical synthesis of the oligo- and polynucleotides of the invention. Procedures for constructing recombinant nucleotide molecules in accordance with the present invention are disclosed in Sambrook, et al., In: Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989), which is incorporated herein in its entirety. Oligonucleotides which encode antisense RNA complementary to senescence-induced deoxyhypusine synthase sequence can be prepared using procedures well known to those in the art. Details concerning such procedures are provided in Maniatis, T. et al., Molecular mechanisms in the Control of Gene expression, eds., Nierlich, et al., eds., Acad. Press, N.Y. (1976).

In an alternative embodiment of the invention, inhibition of expression of endogenous plant senescence-induced DHS, senescence-induced eIF-5A or both is the result of co-suppression through over-expression of an exogenous senescence-induced DHS or eIF-5A gene or gene fragment or both introduced into the plant cell. In this embodiment of the invention, a vector encoding senescence-induced DHS, senescence-induced eIF-5A or both in the sense orientation is introduced into the cells in the same manner as described herein for antisense molecules. Preferably, the senescence-induced DHS or senescence-induced eIF-5A is operatively linked to a strong constitutive promoter, such as for example the fig wart mosaic virus

promoter or CaMV35S or a double 35 S promoter.

In another embodiment of the invention, inhibition of expression of endogenous plant senescence-induced DHS, senescence-induced eIF-5A or both is effected through the use of ribozymes. Ribozymes are RNA molecules exhibiting sequence-specific endoribonuclease activity. An example is the hammerhead ribozyme which cleaves at a UH (where H is an A, C or U residue) recognition site in the target RNA and contains base-pairing regions that direct the catalytic domain of the ribozyme to the target site of the substrate RNA. Ribozymes are highly target-specific and can be designed to inactivate one member of a multigene family or targeted to conserved regions of related mRNAs. (See Merlo et al., *The Plant Cell*, 10:1603-1621, 1998). The ribozyme molecule may be delivered to a cell by transformation, transfection or infection with a vector, such as a plasmid or virus, into which is incorporated the ribozyme operatively linked to appropriate regulatory elements, including a promoter. Such a ribozyme construct contains base-pairing arms that direct it to a cleavage site within the senescence-induced DHS mRNA, or senescence-induced eIF-5A mRNA resulting in cleavage of DHS or eIF-5A mRNA and inhibition of senescence -induced DHSand/or eIF-5A expression.

Transgenic plants made in accordance with the present invention may be prepared by DNA transformation using any method of plant transformation known in the art. Plant transformation methods include direct co-cultivation of plants, tissues or cells with *Agrobacterium tumefaciens* or direct infection (Miki, et al., *Meth. in Plant Mol. Biol. and Biotechnology*, (1993), p. 67-88); direct gene transfer into protoplasts or protoplast uptake (Paszkowski, et al., *EMBO J.*, 12:2717 (1984); electroporation (Fromm, et al., *Nature*, 319:719 (1986); particle bombardment (Klein et al., *BioTechnology*, 6:559-563 (1988); injection into meristematic tissues of seedlings and plants (De LaPena, et al., *Nature*, 325:274-276 (1987); injection into protoplasts of cultured cells and tissues (Reich, et al., *BioTechnology*, 4:1001-1004 (1986)).

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Generally a complete plant is obtained from the transformation process. Plants are regenerated from protoplasts, callus, tissue parts or explants, etc. Plant

parts obtained from the regenerated plants in which the expression of senescence-induced DHS, senescence-induced eIF-5A or both is altered, such as leaves, flowers, fruit, seeds and the like are included in the definition of "plant" as used herein. Progeny, variants and mutants of the regenerated plants are also included  
5 in the definition of "plant."

The tomato, carnation or *Arabidopsis* senescence-induced DHS protein or functional derivatives thereof, and tomato, carnation or *Arabidopsis* senescence-induced eIF-5A protein or functional derivatives thereof are preferably produced by recombinant technologies, optionally in combination with chemical synthesis methods. In one embodiment of the invention the senescence-induced DHS is expressed as a fusion protein, preferably consisting of the senescence-induced DHS fused with maltose binding protein.

"Functional derivatives" of the senescence-induced DHS or senescence-induced eIF-5A protein as described herein are fragments, variants, analogs, or chemical derivatives of senescence-induced DHS or senescence-induced eIF-5A, respectively, which retain at least a portion of the senescence-induced DHS or eIF-5A activity or immunological cross reactivity with an antibody specific for senescence-induced DHS or senescence-induced eIF-5A, respectively. A fragment of the senescence-induced DHS or senescence-induced eIF-5A protein refers to any subset of the molecule. Variant peptides may be made by direct chemical synthesis, for example, using methods well known in the art. An analog of senescence-induced DHS or senescence-induced eIF-5A refers to a non-natural protein substantially similar to either the entire protein or a fragment thereof. Chemical derivatives of senescence-induced DHS or senescence-induced eIF-5A contain additional chemical moieties not normally a part of the peptide or peptide fragment. Modifications may be introduced into peptides or fragments thereof by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues.

A senescence-induced DHS or senescence-induced eIF-5A protein or peptide according to the invention may be produced by culturing a cell transformed with a nucleotide sequence of this invention (in the sense orientation), allowing the cell to synthesize the protein and then isolating the protein, either as a free protein or as a fusion protein, depending on the cloning protocol used, from either the culture medium or from cell extracts. Alternatively, the protein can be produced in a cell-free system. Ranu, et al., Meth. Enzymol., 60:459-484, (1979).

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting to the present invention.

### Example 1

#### Messenger RNA (mRNA) Isolation

Total RNA was isolated from tomato flowers and tomato fruit at various developmental stages and from leaves (untreated or after chilling or sorbitol treatment). Briefly, the tissue (5 g) was ground in liquid nitrogen. The ground powder was mixed with 30 ml guanidinium buffer (4 M guanidinium isothiocyanate, 2.5 mM NaOAc pH 8.5, 0.8% β-mercaptoethanol). The mixture was filtered through four layers of cheesecloth and centrifuged at 10,000 Xg at 4 °C for 30 minutes. The supernatant was then subjected to cesium chloride density gradient centrifugation at 26,000 Xg for 20 hours. The pelleted RNA was rinsed with 75% ethanol, resuspended in 600 µl DEPC-treated water and the RNA precipitated at -70 °C with 0.75 ml 95% ethanol and 30 µl of 3M NaOAc. Ten µg of RNA were fractionated on 25 a 1.2% denaturing formaldehyde agarose gel and transferred to a nylon membrane. Randomly primed <sup>32</sup>P-dCTP-labelled full length DHS cDNA (SEQ ID NO:1) was used to probe the membrane at 42 °C overnight. The membrane was then washed once in 1X SSC containing 0.1% SDS at room temperature for 15 minutes and three times in 0.2X SSC containing 0.1% SDS at 65 °C for 15 minutes each. The 30 membrane was exposed to x-ray film overnight at -70 °C.

PolyA<sup>+</sup> mRNA was isolated from total RNA using the PolyA<sup>+</sup> tract mRNA Isolation System available from Promega. PolyA<sup>+</sup> mRNA was used as a template for

cDNA synthesis using the ZAP Express® cDNA synthesis system available from Stratagene (La Jolla, Calif.)

#### Tomato Leaf cDNA Library Screening

5 A cDNA library made using mRNA isolated from Match F1 hybrid tomato leaves that had been exposed to 2 M sorbitol for six hours was diluted to approximately  $5 \times 10^6$  PFU/ml. The cDNA library was screened using a  $^{32}$ P-labelled 600 bp RT-PCR fragment. Three positive cDNA clones were excised and recircularized into a pBK-CMV® (Stratagene) phagemid using the method in the  
10 manufacturer's instructions. The full length cDNA was inserted into the pBK-CMV vector.

#### Plasmid DNA Isolation, DNA Sequencing

The alkaline lysis method described by Sambrook et al., (Supra) was used to isolate plasmid DNA. The full length positive cDNA clone was sequenced using the dideoxy sequencing method. Sanger, et al., Proc. Natl. Acad. Sci. USA, 74:5463-5467. The open reading frame was compiled and analyzed using BLAST search (GenBank, Bethesda, MD) and alignment of the five most homologous proteins with the derived amino acid sequence of the encoded gene was achieved using a BCM Search Launcher: Multiple Sequence Alignments Pattern-Induced Multiple Alignment Method (See F. Corpet, Nuc. Acids Res., 16:10881-10890, (1987)). Functional motifs present in the derived amino acid sequence were identified by MultiFinder.

#### Northern Blot Hybridizations of Tomato RNA

25 Ten µg of total RNA isolated from tomato flowers at various stages (bud and blossom and senescing petals that are open widely or drying), tomato leaves, and tomato fruit at various stages of ripening (breaker, i.e., green fruit with less than 10% red color, pink, i.e., the entire fruit is orange or pink, and red, either soft or firm) were separated on 1% denatured formaldehyde agarose gels and immobilized on nylon  
30 membranes. The full length tomato cDNA labelled with  $^{32}$ P-dCTP using a random primer kit (Boehringer Mannheim) was used to probe the filters ( $7 \times 10^7$  cpm). The filters were washed once with 1x SSC, 0.1% SDS at room temperature and three

times with 0.2x SSC, 0.1% SDS at 65°C. The filters were dried and exposed to X-ray film overnight at -70°C. The results are shown in Figures 6, 7, 8 and 9.

#### Northern Blot Hybridization of *Arabidopsis* RNA

5 Total RNA from leaves of *Arabidopsis* plants at five weeks of age (lane 1), six weeks (lane 2) and seven weeks (lane 3) was isolated as above, separated on 1% denatured formaldehyde agarose gels and immobilized on nylon membranes. The full-length *Arabidopsis* senescence-induced DHS cDNA labelled with  $^{32}\text{P}$ -dCTP using a random primer kit (Boehringer Mannheim) was used to probe the filters ( $7 \times 10^7$  cpm). The filters were washed once with 1x SSC, 0.1% SDS at room temperature and three times with 0.2x SSC, 0.1% SDS at 65°C. The filters were dried and exposed to X-ray film overnight at -70°C. The results are shown in Figure 11.

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#### Northern Blot Hybridization of Carnation RNA

Total RNA from petals of carnation plants at various stages of flower development, i.e., tight-bud flowers (lane 1), beginning to open (lane 2), fully open flowers (lane 3), flowers with inrolling petals (lane 4), was isolated as above, separated on 1% denatured formaldehyde agarose gels and immobilized on nylon membranes. The full-length carnation senescence-induced DHS cDNA labelled with  $^{32}\text{P}$ -dCTP using a random primer kit (Boehringer Mannheim) was used to probe the filters ( $7 \times 10^7$  cpm). The filters were washed once with 1x SSC, 0.1% SDS at room temperature and three times with 0.2x SSC, 0.1% SDS at 65°C. The filters were dried and exposed to X-ray film overnight at -70°C. The results are shown in Figure 12.

#### **Example 2**

##### Sorbitol Induction of Tomato Senescence-Induced DHS Gene

Tomato leaves were treated with 2 M sorbitol in a sealed chamber for six hours. RNA was extracted from the sorbitol treated leaves as follows.

30 Leaves (5 g) were ground in liquid nitrogen. The ground powder was mixed with 30 ml guanidinium buffer (4 M guanidinium isothiocyanate, 2.5 mM NaOAc pH 8.5, 0.8%  $\beta$ -mercaptoethanol). The mixture was filtered through four layers of

cheesecloth and centrifuged at 10,000 Xg at 4°C for 30 minutes. The supernatant was then subjected to cesium chloride density gradient centrifugation at 26,000 Xg for 20 hours. The pelleted RNA was rinsed with 75% ethanol, resuspended in 600 µl DEPC-treated water and the RNA precipitated at -70°C with 0.75 ml 95% ethanol and 30 µl of 3M NaOAc. Ten µg of RNA were fractionated on a 1.2% denaturing formaldehyde agarose gel and transferred to a nylon membrane. Randomly primed <sup>32</sup>P-dCTP-labelled full length DHS cDNA (SEQ ID NO:1) was used to probe the membrane at 42°C overnight. The membrane was then washed once in 1X SSC containing 0.1% SDS at room temperature for 15 minutes and three times in 0.2X SSC containing 0.1% SDS at 65°C for 15 minutes each. The membrane was exposed to x-ray film overnight at -70°C.

The results are shown in Figure 8. As can be seen, transcription of DHS is induced in leaves by sorbitol.

### Example 3

## Induction of the Tomato DHS gene in Senescing Flowers

Tight flower buds and open, senescing flowers of tomato plants were harvested, and RNA was isolated as in Example 2. Ten µg RNA were fractionated on a 1.2% denaturing formaldehyde agarose gel and transferred to a nylon membrane. Randomly primed <sup>32</sup>P-dCTP-labelled full length DHS cDNA (SEQ ID NO.1) was used to probe the membrane at 42°C overnight. The membrane then was washed once in 1X SSC containing 0.1% SDS at room temperature for 15 minutes and then washed three times in 0.2X SSC containing 0.1% SDS at 65°C for fifteen minutes each. The membrane was exposed to x-ray film overnight at -70°C.

The results are shown in Figure 6. As can be seen, transcription of DHS is induced in senescent flowers.

30 Example 4

## Induction of the Tomato DHS Gene in Ripening Fruit

RNA was isolated from breaker, pink and ripe fruit as in Example 2. Ten µg

RNA were fractionated on a 1.2% denaturing formaldehyde agarose gel and transferred to a nylon membrane. Randomly primed  $^{32}\text{P}$ -dCTP-labelled full length DHS cDNA (SEQ ID NO.1) (Figure 1) was used to probe the membrane at 42°C overnight. The membrane then was washed once in 1X SSC containing 0.1% SDS at room temperature for 15 minutes and then washed three times in 0.2X SSC containing 0.1% SDS at 65°C for fifteen minutes each. The membrane was exposed to x-ray film overnight at -70°C.

The results are shown in Figure 7. As can be seen, transcription of DHS is strongest in ripe, red fruit just prior to the onset of senescence leading to spoilage.

### **Example 5**

#### Induction of Tomato Senescence-Induced DHS Gene by Chilling

Tomato plants in pots (7-8 weeks old) were exposed to 6°C for two days, three days or six days in a growth chamber. The light cycle was set for eight hours of dark and sixteen hours of light. Plants were rewarmed by moving them back into a greenhouse. Plants that were not rewarmed were harvested immediately after removal from the growth chamber. RNA was extracted from the leaves as follows.

Leaves (5 g) were ground in liquid nitrogen. The ground powder was mixed with 30 ml guanidinium buffer (4 M guanidinium isothiocyanate, 2.5 mM NaOAc pH 8.5, 0.8%  $\beta$ -mercaptoethanol). The mixture was filtered through four layers of cheesecloth and centrifuged at 10,000g at 4°C for 30 minutes. The supernatant was then subjected to cesium chloride density gradient centrifugation at 26,000g for 20 hours. The pelleted RNA was rinsed with 75% ethanol, resuspended in 600  $\mu\text{l}$  DEPC-treated water and the RNA precipitated at -70°C with 0.75 ml 95% ethanol and 30  $\mu\text{l}$  of 3M NaOAc. Ten  $\mu\text{g}$  of RNA were fractionated on a 1.2% denaturing formaldehyde agarose gel and transferred to a nylon membrane. Randomly primed  $^{32}\text{P}$ -dCTP-labelled full length DHS cDNA (SEQ ID NO:1) was used to probe the membrane at 42°C overnight. The membrane was then washed once in 1X SSC containing 0.1% SDS at room temperature for 15 minutes and three times in 0.2X SSC containing 0.1% SDS at 65°C for 15 minutes each. The membrane was exposed to x-ray film overnight at -70°C.

The results are shown in Figure 9. As can be seen, transcription of DHS is induced in leaves by exposure to chilling temperature and subsequent rewarming, and the enhanced transcription correlates with chilling damage measured as membrane leakiness.

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### **Example 6**

#### Generation of an *Arabidopsis* PCR Product Using Primers Based on Unidentified *Arabidopsis* Genomic Sequence

A partial length senescence-induced DHS sequence from an *Arabidopsis* cDNA template was generated by PCR using a pair of oligonucleotide primers designed from *Arabidopsis* genomic sequence. The 5' primer is a 19-mer having the sequence, 5'- GGTGGTGT5TGAGGAAGATC (SEQ ID NO:7); the 3' primer is a 20 mer having the sequence, GGTGCACGCCCTGATGAAGC -3' (SEQ ID NO:8). A polymerase chain reaction using the Expand High Fidelity PCR System (Boehringer Mannheim) and an *Arabidopsis* senescent leaf cDNA library as template was carried out as follows.

#### Reaction components:

cDNA	1 $\mu$ l (5 $\times$ 10 <sup>7</sup> pfu)
dNTP (10 mM each)	1 $\mu$ l
MgCl <sub>2</sub> (5mM)+10x buffer	5 $\mu$ l
Primers 1 and 2 (100 $\mu$ M each)	2 $\mu$ l
Expand High Fidelity DNA polymerase	1.75 U
Reaction volume	50 $\mu$ l

#### Reaction parameters:

94°C for 3 min  
94°C /1 min, 58°C /1 min, 72°C /2 min, for 45 cycles  
72°C for 15 min .

25

### **Example 7**

#### Isolation of Genomic DNA and Southern Analysis

Genomic DNA was extracted from tomato leaves by grinding 10 grams of tomato leaf tissue to a fine powder in liquid nitrogen. 37.5 ml of a mixture containing 25 ml homogenization buffer [100 mM Tris-HCl, pH 8.0, 100 mM EDTA, 250 mM NaCl, 1% sarkosyl, 1% 2-mercaptoethanol, 10  $\mu$ g/ml RNase and 12.5 ml phenol]

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prewarmed to 60°C was added to the ground tissue. The mixture was shaken for fifteen minutes. An additional 12.5 ml of chloroform/isoamyl alcohol (24:1) was added to the mixture and shaken for another 15 minutes. The mixture was centrifuged and the aqueous phase reextracted with 25 ml  
5 phenol/chloroform/isoamylalcohol (25:24:1) and chloroform/ isoamylalcohol (24:1). The nucleic acids were recovered by precipitaion with 15 ml isopropanol at room temperature. The precipitate was resuspended in 1 ml of water.

Genomic DNA was subjected to restriction enzyme digestion as follows:  
10 10 µg genomic DNA, 40 µl 10X reaction buffer and 100 U restriction enzyme (XbaI, EcoRI, EcoRV or HinDIII) were reacted for five to six hours in a total reaction volume of 400 µl. The mixture was then phenol-extracted and ethanol-precipitated. The digested DNA was subjected to agarose gel electrophoresis on a 0.8% agarose gel at 15 volts for approximately 15 hours. The gel was submerged in denaturation buffer [87.66 g NaCl and 20 g NaOH /Liter] for 30 minutes with gentle agitation, rinsed in distilled water and submerged in neutralization buffer [87.66 g NaCl and 60.55 g tris-HCl, pH 7.5/Liter] for 30 minutes with gentle agitation. The DNA was transferred to a Hybond-N<sup>+</sup> nylon membrane by capillary blotting.

Hybridization was performed overnight at 42°C using  $1 \times 10^6$  cpm/ml of <sup>32</sup>P-dCTP-labeled full length DHS cDNA or 3'-non-coding region of the DHS cDNA clone. Prehybridization and hybridization were carried out in buffer containing 50% formamide, 6X SSC, 5X Denhardt's solution, 0.1% SDS and 100 mg/ml denatured salmon sperm DNA. The membrane was prehybridized for two to four hours;  
25 hybridization was carried out overnight.

After hybridization was complete, membranes were rinsed at room temperature in 2X SSC and 0.1% SDS and then washed in 2X SSC and 0.1% SDS for 15 minutes and 0.2X SSC and 0.1% SDS for 15 minutes. The membrane was then exposed to x-ray film at -80°C overnight. The results are shown in Figure 5.  
30

### Example 8

**Isolation Of A Senescence-Induced eIF-5A Gene From *Arabidopsis***

A full-length cDNA clone of the senescence-induced eIF-5A gene expressed in *Arabidopsis* leaves was obtained by PCR using an *Arabidopsis* senescent leaf cDNA library as template. Initially, PCR products corresponding to the 5'- and 3'- ends of the gene were made using a degenerate upstream primer  
5 <AAARRYCGMCCYTGCAAGGT>(SEQ ID NO:17) paired with vector T7 primer  
<AATACGACTCACTATAG> (SEQ ID NO:18), and a degenerate downstream primer  
<TCYTTNCCYTCMKCTAAHCC> (SEQ ID NO:19) paired with vector T3 primer  
<ATTAACCCTCACTAAAG> (SEQ ID NO: 20). The PCR products were subcloned  
10 into pBluescript for sequencing. The full-length cDNA was then obtained using a 5'- specific primer <CTGTTACCAAAAAATCTGTACC> (SEQ ID NO: 21) paired with a 3'-specific primer <AGAAGAAGTATAAAAACCATC> (SEQ ID NO: 22), and subcloned into pBluescript for sequencing.

**Example 9**

**Isolation Of A Senescence-Induced eIF-5A Gene From Tomato Fruit**

A full-length cDNA clone of the senescence-induced eIF-5A gene expressed in tomato fruit was obtained by PCR using a tomato fruit cDNA library as template. Initially, PCR products corresponding to the 5'- and 3'- ends of the gene were made using a degenerate upstream primer (SEQ ID NO:17) paired with vector T7 primer (SEQ ID NO:18), and a degenerate downstream primer (SEQ ID NO:19) paired with vector T3 primer (SEQ ID NO: 20). The PCR products were subcloned into pBluescript for sequencing. The full-length cDNA was then obtained using a 5'- specific primer <AAAGAACCTAGAGAGAGAAAGG> (SEQ ID NO: 23) paired with vector T7 primer (SEQ ID NO: 18), and subcloned into pBluescript for sequencing.  
25

**Example 10**

**Isolation Of A Senescence-Induced eIF-5A Gene From Carnation**

A full-length cDNA clone of the senescence-induced eIF-5A gene expressed in carnation flowers was obtained by PCR using a carnation senescent flower cDNA library as template. Initially, PCR products corresponding to the 5'- and 3'- ends of the gene were made using a degenerate upstream primer (SEQ ID NO:17) paired  
30

with vector T7 primer (SEQ ID NO:18), and a degenerate downstream primer (SEQ ID NO:19) paired with vector T3 primer (SEQ ID NO: 20). The PCR products were subcloned into pBluescript for sequencing. The full-length cDNA was then obtained using a 5'-specific primer <TTTTACATCAATCGAAAA> (SEQ ID NO: 24) paired with 5 a 3'-specific primer <ACCAAAACCTGTGTTAACTCC> (SEQ ID NO: 25), and subcloned into pBluescript for sequencing.

### **Example 11**

#### Isolation Of A Senescence-Induced DHS Gene From *Arabidopsis*

A full-length cDNA clone of the senescence-induced DHS gene expressed in *Arabidopsis* leaves was obtained by screening an *Arabidopsis* senescing leaf cDNA library. The sequence of the probe (SEQ ID NO: 26) that was used for screening is shown in Figure 38. The probe was obtained by PCR using the senescence leaf cDNA library as a template and primers (indicated as underlined regions in Figure 38) designed from the unidentified genomic sequence (AB017060) in GenBank. The PCR product was subcloned into pBluescript for sequencing.

### **Example 12**

#### Isolation Of A Senescence-Induced DHS Gene From Carnation

A full-length cDNA clone of the senescence-induced DHS gene expressed in carnation petals was obtained by screening a carnation senescing petal cDNA library. The sequence of the probe (SEQ ID NO: 27) that was used for screening is shown in Figure 39. The probe was obtained by PCR using the senescence petal cDNA library as a template and degenerate primers (upstream: 5' TTG ARG AAG ATY CAT MAA RTG CCT 3') (SEQ ID NO: 28); downstream: 5' CCA TCA AAY TCY TGK GCR GTG TT 3') (SEQ ID NO: 29)). The PCR product was subcloned into pBluescript for sequencing.

### **Example 13**

#### Transformation Of *Arabidopsis* With Full-Length Or 3' Region Of *Arabidopsis* DHS In Antisense Orientation

SEQUENCE DRAFT

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*Agrobacteria* were transformed with the binary vector, pKYLX71, containing the full-length senescence-induced *Arabidopsis* DHS cDNA sequence or the 3' end of the DHS gene (SEQ ID NO:30) (Figure 36), both expressed in the antisense configuration, under the regulation of double 35S promoter. *Arabidopsis* plants were transformed with the transformed *Agrobacteria* by vacuum infiltration, and transformed seeds from resultant T<sub>0</sub> plants were selected on ampicillin.

Figures 21 through 24 are photographs of the transformed *Arabidopsis* plants, showing that expression of the DHS gene or 3' end thereof in antisense orientation in the transformed plants results in increased biomass, e.g., larger leaves and increased plant size. Figure 25 illustrates that the transgenic *Arabidopsis* plants have increased seed yield.

#### **Example 14**

##### Transformation Of Tomato Plants With Full-Length Or 3' Region Of Tomato DHS In Antisense Orientation

*Agrobacteria* were transformed with the binary vector, pKYLX71, containing the full-length senescence-induced tomato DHS cDNA sequence or the 3' end of the DHS gene (SEQ ID NO:31) (Figure 37), both expressed in the antisense configuration, under the regulation of double 35S promoter. Tomato leaf explants were formed with these *Agrobacteria*, and transformed callus and plantlets were generated and selected by standard tissue culture methods. Transformed plantlets were grown to mature fruit-producing T<sub>1</sub> plants under greenhouse conditions.

Figures 26 through 35 are photographs showing that reduced expression of the senescence-induced tomato DHS gene in the transformed plants results in increased biomass, e.g., larger leaf size and larger plants as seen in the transformed *Arabidopsis* plants, as well as delayed softening and spoilage of tomato fruit.

#### **Example 15**

##### Transformation of Tomato Plants With the 3' Region of Tomato DHS in Anitsense

## Orientation

Agrobacteria were transformed with the binary vector, pKYLX71, containing the 3'end of the DHS gene (Figure 37) expressed in the antisense configuration, under the regulation of double 35S promoter. Tomato leaf explants were formed with 5 these Agrobacteria, and transformed callus and plantlets were generated and selected by standard tissue culture methods. Transformed plantlets were grown to mature fruit producing T<sub>1</sub> plants under green house conditions.

Fruit from these transgenic plants with reduced DHS expression were 10 completely free of blossom end rot under conditions in which about 33% of fruit from control plants developed this disease. Blossom end rot is a physiological disease attributable to nutrient stress that causes the bottom (blossom) end of the fruit to senesce and rot. Figures 40(a) and 40(b) are photographs showing a control fruit exhibiting blossom end rot and a transgenic fruit that is free of blossom end rot.

The results indicate that reducing the expression of DHS prevents the onset of tissue and cell death arising from physiological disease.